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TITLE OF THE INVENTION

**Nucleotide sequences which code for the metE gene**

BACKGROUND OF THE INVENTION

5 Field of the Invention

The invention provides nucleotide sequences from coryneform bacteria which code for the metE gene and a process for the fermentative preparation of amino acids, in particular L-methionine, using bacteria in which the metE gene is enhanced.

10 Description of the Related Art

L-Amino acids, in particular L-methionine, are used in human medicine and in the pharmaceuticals industry, in the foodstuffs industry and very particularly in animal nutrition.

It is known that amino acids are prepared by fermentation from strains of coryneform bacteria, in particular *Corynebacterium glutamicum*. Because of their great importance, work is constantly being undertaken to improve the preparation process. Improvements to the process can relate to fermentation measures stirring and supply of oxygen, or to the composition of the nutrient media such as the sugar concentration during the fermentation, or to the working up of the product by, for example, ion exchange chromatography, or to the intrinsic output properties of the microorganism itself.

25 Methods of mutagenesis, selection and mutant selection are used to improve the output properties of these microorganisms. Strains which are resistant to antimetabolites, such as e.g. the methionine analogue  $\alpha$ -methyl-methionine, ethionine, norleucine, N-acetylnorleucine, S-trifluoromethylhomocysteine,  
30 2-amino-5-heprenoitic acid, seleno-methionine, methionine-sulfoximine, methoxine, 1-aminocyclopentane-carboxylic acid, or are auxotrophic for metabolites of regulatory importance

and produce amino acids, such as e.g. L-methionine, are obtained in this manner.

Recombinant DNA techniques have also been employed for some years for improving the Corynebacterium strains which produce L-amino acids, by amplifying individual amino acid biosynthesis genes and investigating their effect on the amino acid production.

#### SUMMARY OF THE INVENTION

10 An object of the present invention is to provide new measures for improved fermentative preparation of amino acids, in particular L-methionine.

15 When L-methionine or methionine are mentioned in the following, the salts, such as methionine hydrochloride or methionine sulfate are also meant.

The invention provides an isolated polynucleotide from coryneform bacteria, comprising a polynucleotide sequence which codes for the metE gene, chosen from the group consisting of

- 20 a) polynucleotide which is at least 70% identical to a polynucleotide that codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 2,
- b) polynucleotide which codes for a polypeptide that comprises an amino acid sequence which is at least 70% identical to the amino acid sequence of SEQ ID No. 2,
- 25 c) polynucleotide which is complementary to the polynucleotides of a) or b), and
- d) polynucleotide comprising at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c),
- 30 and the corresponding polypeptide having the enzymatic activity of homocysteine methyltransferase I.

The invention also provides the above-mentioned polynucleotides as DNA which is capable of replication, comprising:

- (i) the nucleotide sequence shown in SEQ ID No. 1, or
- 5 (ii) at least one sequence which corresponds to sequence (i) within the range of the degeneration of the genetic code, or
- (iii) at least one sequence which hybridizes with the sequence complementary to sequence (i) or (ii), and
- 10 (iv) sense mutations of neutral function in (i).

The invention also provides

a polynucleotide comprising the nucleotide sequence as shown in SEQ ID No. 1;

15 a polynucleotide that codes for a polypeptide which comprises the amino acid sequence as shown in SEQ ID No. 2;

a vector containing the polynucleotide according to the invention, in particular a shuttle vector or plasmid vector, and

20 and coryneform bacteria serving as the host cell, which contain the vector or in which the metE gene is enhanced.

The invention also provides polynucleotides which are obtained by screening a corresponding gene library, which comprises the complete gene having the polynucleotide sequence corresponding

25 to SEQ ID No. 1, by means of hybridization with a probe which comprises the sequence of the polynucleotide mentioned, according to SEQ ID No. 1 or a fragment thereof, and isolation of the DNA sequence mentioned.

#### BRIEF DESCRIPTION OF THE FIGURES

30 Fig. 1 shows plasmid pCREmetAE.

Fig. 2 shows plasmid pCREmetAEY.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Polynucleotides which comprise the sequences according to the invention are suitable as hybridization probes for RNA, cDNA and DNA, in order to isolate, in the full length, nucleic acids or polynucleotides or genes which code for homocysteine methyltransferase I or to isolate those nucleic acids or polynucleotides or genes which have a high similarity of sequence or homology with that of the homocysteine methyltransferase I gene.

Polynucleotides according to the invention are furthermore suitable as primers with which the DNA of genes that code for homocysteine methyltransferase I can be prepared by the polymerase chain reaction (PCR).

Such oligonucleotides that serve as probes or primers comprise at least 30, preferably at least 20, very particularly at least 15 successive nucleotides. Oligonucleotides which have a length of at least 40 or 50 nucleotides are also suitable. Oligonucleotides with a length of at least 100, 150, 200, 250 or 300 nucleotides are optionally also suitable.

"Isolated" means separated out of its natural environment.

"Polynucleotide" in general relates to polyribonucleotides and polydeoxyribonucleotides, it being possible for these to be non-modified RNA or DNA or modified RNA or DNA.

"Polypeptides" are understood as meaning peptides or proteins which comprise two or more amino acids bonded via peptide bonds.

The polypeptides according to the invention include a polypeptide according to SEQ ID No. 2, in particular those with the biological activity of homocysteine methyltransferase I, and also those which are at least 70%, preferably at least 80% and in particular at least 90% to 95% identical to the

polypeptide according to SEQ ID No. 2 and have the activity mentioned.

The invention moreover provides a process for the fermentative preparation of amino acids, in particular L-methionine, using coryneform bacteria which in particular already produce amino acids, and in which the nucleotide sequences which code for the metE gene are enhanced, in particular over-expressed.

The term "enhancement" in this connection describes an increase in the intracellular activity of one or more enzymes (proteins) in a microorganism which are coded by the corresponding DNA, for example by increasing the number of copies of the gene or genes, using a potent promoter or using a gene or allele which codes for a corresponding enzyme (protein) having a high activity, and optionally combining these measures.

By enhancement measures, in particular over-expression, the activity or concentration of the corresponding protein is in general increased by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, up to a maximum of 1000% or 2000%, based on the starting microorganism.

The microorganisms which the present invention provides can prepare L-amino acids, in particular L-methionine, from glucose, sucrose, lactose, fructose, maltose, molasses, starch, cellulose or from glycerol and ethanol. They can be representatives of coryneform bacteria, in particular of the genus *Corynebacterium*. Of the genus *Corynebacterium*, there may be mentioned in particular the species *Corynebacterium glutamicum*, which is known among experts for its ability to produce L-amino acids.

Suitable strains of the genus *Corynebacterium*, in particular of the species *Corynebacterium glutamicum* (*C. glutamicum*), are in particular the known wild-type strains

*Corynebacterium glutamicum* ATCC13032

*Corynebacterium acetoglutamicum* ATCC15806

Corynebacterium acetoacidophilum ATCC13870  
Corynebacterium thermoaminogenes FERM BP-1539  
Corynebacterium melassecola ATCC17965  
Brevibacterium flavum ATCC14067  
5 Brevibacterium lactofermentum ATCC13869 and  
Brevibacterium divaricatum ATCC14020

or L-amino acid-producing mutants or strains prepared  
therefrom, such as, for example, the L-methionine-producing  
strain

10 Corynebacterium glutamicum ATCC21608.

The new metE gene from C. glutamicum which codes for the  
enzyme homocysteine methyltransferase I (EC 2.1.1.14) has been  
isolated.

15 To isolate the metE gene or also other genes of C. glutamicum,  
a gene library of this microorganism is first set up in  
Escherichia coli (E. coli). The setting up of gene libraries  
is described in generally known textbooks and handbooks. The  
textbook by Winnacker: Gene und Klone, Eine Einführung in die  
Gentechnologie (Verlag Chemie, Weinheim, Germany, 1990), or  
20 the handbook by Sambrook et al.: Molecular Cloning, A  
Laboratory Manual (Cold Spring Harbor Laboratory Press, 1989)  
may be mentioned as example. A well-known gene library is that  
of the E. coli K-12 strain W3110 set up in  $\lambda$  vectors by Kohara  
et al. (Cell 50, 495 -508 (1987)). Bathe et al. (Molecular and  
25 General Genetics, 252:255-265, 1996) describe a gene library  
of C. glutamicum ATCC13032, which was set up with the aid of  
the cosmid vector SuperCos I (Wahl et al., 1987, Proceedings  
of the National Academy of Sciences USA, 84:2160-2164) in the  
E. coli K-12 strain NM554 (Raleigh et al., 1988, Nucleic Acids  
30 Research 16:1563-1575).

Börmann et al. (Molecular Microbiology 6(3), 317-326) (1992))  
in turn describe a gene library of C. glutamicum ATCC13032  
using the cosmid pHC79 (Hohn and Collins, Gene 11, 291-298  
(1980)). To prepare a gene library of C. glutamicum in E. coli  
35 it is also possible to use plasmids such as pBR322 (Bolivar,

Life Sciences, 25, 807-818 (1979)) or pUC9 (Vieira et al., 1982, Gene, 19:259-268). Suitable hosts are, in particular, those E. coli strains which are restriction- and recombination-defective. An example of these is the strain DH5 $\alpha$ mc<sup>r</sup>, which has been described by Grant et al. (Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649). The long DNA fragments cloned with the aid of cosmids can in turn be subcloned in the usual vectors suitable for sequencing and then sequenced, as is described e.g. by Sanger et al. (Proceedings of the National Academy of Sciences of the United States of America, 74:5463-5467, 1977).

The resulting DNA sequences can then be investigated with known algorithms or sequence analysis programs, such as that of Staden (Nucleic Acids Research 14, 217-232(1986)), that of Marck (Nucleic Acids Research 16, 1829-1836 (1988)) or the GCG program of Butler (Methods of Biochemical Analysis 39, 74-97 (1998)).

The new DNA sequence of C. glutamicum which codes for the metE gene and which, as SEQ ID No. 1, is a constituent of the present invention has been found. The amino acid sequence of the corresponding protein has furthermore been derived from the present DNA sequence by the methods described above. The resulting amino acid sequence of the metE gene product is shown in SEQ ID No. 2.

Coding DNA sequences which result from SEQ ID No. 1 by the degeneracy of the genetic code are also a constituent of the invention. In the same way, DNA sequences which hybridize with SEQ ID No. 1 or parts of SEQ ID No. 1 are a constituent of the invention. Conservative amino acid exchanges, such as e.g. exchange of glycine for alanine or of aspartic acid for glutamic acid in proteins, are furthermore known among experts as "sense mutations" which do not lead to a fundamental change in the activity of the protein, i.e. they are of neutral function.

It is furthermore known that changes at the N and/or C terminus of a protein must not substantially impair and may

even stabilize the function thereof. Information in this context can be found in Ben-Bassat et al. (Journal of Bacteriology 169:751-757 (1987)), in O'Regan et al. (Gene 77:237-251 (1989)), in Sahin-Toth et al. (Protein Sciences 3:240-247 (1994)), in Hochuli et al. (Bio/Technology 6:1321-1325 (1988)) and in known textbooks of genetics and molecular biology. Amino acid sequences which result in a corresponding manner from SEQ ID No. 2 are also a constituent of the invention.

In the same way, DNA sequences which hybridize with SEQ ID No. 1 or parts of SEQ ID No. 1 are a constituent of the invention. Finally, DNA sequences which are prepared by the polymerase chain reaction (PCR) using primers which result from SEQ ID No. 1 are a constituent of the invention. Such oligonucleotides typically have a length of at least 15 nucleotides.

Instructions for identifying DNA sequences by means of hybridization can be found in the handbook "The DIG System Users Guide for Filter Hybridization" from Boehringer Mannheim GmbH (Mannheim, Germany, 1993) and in Liebl et al. (International Journal of Systematic Bacteriology (1991) 41: 255-260). Instructions for amplification of DNA sequences with the aid of the polymerase chain reaction (PCR) can be found in the handbook by Gait: Oligonucleotide synthesis: A Practical Approach (IRL Press, Oxford, UK, 1984) and in Newton and Graham: PCR (Spektrum Akademischer Verlag, Heidelberg, Germany, 1994).

It has been found that coryneform bacteria produce amino acids, in particular L-methionine, in an improved manner after over-expression of the metE gene.

To achieve an over-expression, the number of copies of the corresponding genes can be increased, or the promoter and regulation region or the ribosome binding site upstream of the structural gene can be mutated. Expression cassettes which are incorporated upstream of the structural gene act in the same way. By inducible promoters, it is additionally possible to



increase the expression in the course of fermentative L-methionine production. The expression is likewise improved by measures to prolong the life of the m-RNA. Furthermore, the enzyme activity is also increased by preventing the degradation of the enzyme protein. The genes or gene constructs can either be present in plasmids with a varying number of copies, or can be integrated and amplified in the chromosome. Alternatively, an over-expression of the genes in question can furthermore be achieved by changing the composition of the media and the culture procedure.

Instructions in this context can be found in Martin et al. (Bio/Technology 5, 137-146 (1987)), in Guerrero et al. (Gene 138, 35-41 (1994)), Tsuchiya and Morinaga (Bio/Technology 6, 428-430 (1988)), in Eikmanns et al. (Gene 102, 93-98 (1991)), in European Patent Specification 0 472 869, in US Patent 4,601,893, in Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991)), in Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)), in LaBarre et al. (Journal of Bacteriology 175, 1001-1007 (1993)), in Patent Application WO 96/15246, in Malumbres et al. (Gene 134, 15 - 24 (1993)), in Japanese Laid-Open Specification JP-A-10-229891, in Jensen and Hammer (Biotechnology and Bioengineering 58, 191-195 (1998)), in Makrides (Microbiological Reviews 60:512-538 (1996)) and in known textbooks of genetics and molecular biology.

By way of example, for enhancement the metE gene according to the invention was over-expressed with the aid of episomal plasmids. Suitable plasmids are those which are replicated in coryneform bacteria. Numerous known plasmid vectors, such as e.g. pZ1 (Menkel et al., Applied and Environmental Microbiology (1989) 64: 549-554), pEKEx1 (Eikmanns et al., Gene 102:93-98 (1991)) or pHS2-1 (Sonnen et al., Gene 107:69-74 (1991)) are based on the cryptic plasmids pHM1519, pBL1 or pGA1. Other plasmid vectors, such as those based on pCG4 (US-A 4,489,160), or pNG2 (Serwold-Davis et al., FEMS Microbiology Letters 66, 119-124 (1990)), or pAG1 (US-A 5,158,891), can be used in the same manner.

Plasmid vectors which are furthermore suitable are also those with the aid of which the process of gene amplification by integration into the chromosome can be used, as has been described, for example, by Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)) for duplication or amplification of the hom-thrB operon. In this method, the complete gene is cloned in a plasmid vector which can replicate in a host (typically E. coli), but not in C. glutamicum. Possible vectors are, for example, pSUP301 (Simon et al., Bio/Technology 1, 784-791 (1983)), pK18mob or pK19mob (Schäfer et al., Gene 145, 69-73 (1994)), pGEM-T (Promega corporation, Madison, WI, USA), pCR2.1-TOPO (Shuman (1994). Journal of Biological Chemistry 269:32678-84; US-A 5,487,993), pCR®Blunt (Invitrogen, Groningen, Holland; Bernard et al., Journal of Molecular Biology, 234: 534-541 (1993)), pEM1 (Schrumpf et al, 1991, Journal of Bacteriology 173:4510-4516) or pBGS8 (Spratt et al., 1986, Gene 41: 337-342). The plasmid vector which contains the gene to be amplified is then transferred into the desired strain of C. glutamicum by conjugation or transformation. The method of conjugation is described, for example, by Schäfer et al. (Applied and Environmental Microbiology 60, 756-759 (1994)). Methods for transformation are described, for example, by Thierbach et al. (Applied Microbiology and Biotechnology 29, 356-362 (1988)), Dunican and Shivnan (Bio/Technology 7, 1067-1070 (1989)) and Tauch et al. (FEMS Microbiological Letters 123, 343-347 (1994)). After homologous recombination by means of a "cross over" event, the resulting strain contains at least two copies of the gene in question.

In addition, it may be advantageous for the production of amino acids, in particular L-methionine, to enhance one or more enzymes of the particular biosynthesis pathway, of glycolysis, of anaplerosis, of the citric acid cycle or of amino acid export, in addition to the metE gene.

Thus for the preparation of amino acids, in particular L-methionine, one or more genes chosen from the group consisting of

- the gap gene which codes for glyceraldehyde 3-phosphate dehydrogenase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- 5 • the tpi gene which codes for triose phosphate isomerase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the pgk gene which codes for 3-phosphoglycerate kinase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the pyc gene which codes for pyruvate carboxylase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- 10 • the lysC gene which codes for a feed-back resistant aspartate kinase (Accession No.P26512; EP-B-0387527; EP-A-0699759),
- the metaA gene which codes for homoserine O-acetyltransferase (ACCESSION Number AF052652),
- 15 • the metB gene which codes for cystathionine gamma-synthase (ACCESSION Number AF126953),
- the aecD gene which codes for cystathionine gamma-lyase (ACCESSION Number M89931)
- the glyA gene which codes for serine hydroxymethyltransferase (JP-A-08107788),
- 20 • the metY gene which codes for O-acetylhomoserine sulfhydrylase (DSM 13556)

can be enhanced, in particular over-expressed.

25 It may furthermore be advantageous for the production of amino acids, in particular L-methionine, in addition to the enhancement of the metE gene, for one or more genes chosen from the group consisting of

- the thrB gene which codes for homoserine kinase (ACCESSION Number P08210),

- the *ilvA* gene which codes for threonine dehydratase (ACCESSION Number Q04513),
- the *thrC* gene which codes for threonine synthase (ACCESSION Number P23669),
- 5 • the *ddh* gene which codes for meso-diaminopimelate D-dehydrogenase (ACCESSION Number Y00151),
- the *pck* gene which codes for phosphoenol pyruvate carboxykinase (DE 199 50 409.1; DSM 13047),
- 10 • the *pgi* gene which codes for glucose 6-phosphate isomerase (US 09/396,478; DSM 12969),
- the *poxB* gene which codes for pyruvate oxidase (DE: 1995 1975.7; DSM 13114)

to be attenuated, in particular for the expression thereof to be reduced.

15 The term "attenuation" in this connection describes the reduction or elimination of the intracellular activity of one or more enzymes (proteins) in a microorganism which are coded by the corresponding DNA, for example by using a weak promoter or using a gene or allele which codes for a corresponding  
20 enzyme with a low activity or inactivates the corresponding gene or enzyme (protein), and optionally combining these measures.

By attenuation measures, the activity or concentration of the corresponding protein is in general reduced to 0 to 50%, 0 to  
25 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild-type protein.

In addition to over-expression of the *metE* gene it may furthermore be advantageous for the production of amino acids, in particular L-methionine, to eliminate undesirable side  
30 reactions, (Nakayama: "Breeding of Amino Acid Producing Micro-organisms", in: Overproduction of Microbial Products,

Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

5 The microorganisms prepared according to the invention can be  
cultured continuously or discontinuously in the batch process  
(batch culture) or in the fed batch (feed process) or repeated  
fed batch process (repetitive feed process) for the purpose of  
production of amino acids, in particular L-methionine. A  
summary of known culture methods is described in the textbook  
by Chmiel (Bioprozesstechnik 1. Einführung in die  
10 Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991))  
or in the textbook by Storhas (Bioreaktoren und periphere  
Einrichtungen (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

15 The culture medium to be used must meet the requirements of  
the particular strains in a suitable manner. Descriptions of  
culture media for various microorganisms are contained in the  
handbook "Manual of Methods for General Bacteriology" of the  
American Society for Bacteriology (Washington D.C., USA,  
1981).

20 Sugars and carbohydrates, such as e.g. glucose, sucrose,  
lactose, fructose, maltose, molasses, starch and cellulose,  
oils and fats, such as e.g. soya oil, sunflower oil, groundnut  
oil and coconut fat, fatty acids, such as e.g. palmitic acid,  
stearic acid and linoleic acid, alcohols, such as e.g.  
glycerol and ethanol, and organic acids, such as e.g. acetic  
25 acid, can be used as the source of carbon. These substance can  
be used individually or as a mixture.

30 Organic nitrogen-containing compounds, such as peptones, yeast  
extract, meat extract, malt extract, corn steep liquor, soya  
bean flour and urea, or inorganic compounds, such as ammonium  
sulfate, ammonium chloride, ammonium phosphate, ammonium  
carbonate and ammonium nitrate, can be used as the source of  
nitrogen. The sources of nitrogen can be used individually or  
as a mixture.

35 Organic and inorganic sulfur-containing compounds, such as,  
for example, sulfides, sulfites, sulfates and thiosulfates,

can be used as a source of sulfur, in particular for the preparation of methionine.

Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus. The culture medium must furthermore comprise salts of metals, such as e. g. magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be employed in addition to the above-mentioned substances. Suitable precursors can moreover be added to the culture medium. The starting substances mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH of the culture. Antifoams, such as e.g. fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, such as e.g. antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as e.g. air, are introduced into the culture. The temperature of the culture is usually 20°C to 45°C, and preferably 25°C to 40°C. Culturing is continued until a maximum of the desired product has formed. This target is usually reached within 10 hours to 160 hours.

The fermentation broths obtained in this way, in particular containing L-methionine, usually have a dry weight of 7.5 to 25 wt.% and contain L-methionine. It is furthermore also advantageous if the fermentation is conducted in a sugar-limited procedure at least at the end, but in particular over at least 30% of the duration of the fermentation. That is to say, the concentration of utilizable sugar in the fermentation medium is reduced to  $\geq 0$  to 3 g/l during this period.

The fermentation broth prepared in this manner, in particular containing L-methionine, is then further processed. Depending on requirements all or some of the biomass can be removed from the fermentation broth by separation methods, such as centrifugation, filtration, decanting or a combination thereof, or it can be left completely in. This broth is then thickened or concentrated by known methods, such as with the aid of a rotary evaporator, thin film evaporator, falling film evaporator, by reverse osmosis, or by nanofiltration. This concentrated fermentation broth can then be worked up by methods of freeze drying, spray drying, spray granulation or by other processes to give a preferably free-flowing, finely divided powder.

This free-flowing, finely divided powder can then in turn be converted by suitable compacting or granulating processes into a coarse-grained, readily free-flowing, storable and largely dust-free product. In the granulation or compacting it is advantageous to employ conventional organic or inorganic auxiliary substances or carriers, such as starch, gelatin, cellulose derivatives or similar substances, such as are conventionally used as binders, gelling agents or thickeners in foodstuffs or feedstuffs processing, or further substances, such as, for example, silicas, silicates or stearates.

"Free-flowing" is understood as meaning powders which flow unimpeded out of the vessel with the opening of 5 mm (millimeters) of a series of glass outflow vessels with outflow openings of various sizes (Klein, Seifen, Öle, Fette, Wachse 94, 12 (1968)).

As described here, "finely divided" means a powder with a predominant content (> 50 %) having a particle size of 20 to 200  $\mu\text{m}$  diameter. "Coarse-grained" means products with a predominant content (> 50 %) having a particle size of 200 to 2000  $\mu\text{m}$  diameter. In this context, "dust-free" means that the product contains only small contents (< 5 %) having particle sizes of less than 20  $\mu\text{m}$  diameter. The particle size determination can be carried out with methods of laser

diffraction spectrometry. The corresponding methods are described in the textbook on "Teilchengrößenmessung in der Laborpraxis" by R. H. Müller and R. Schuhmann, Wissenschaftliche Verlagsgesellschaft Stuttgart (1996) or in the textbook "Introduction to Particle Technology" by M. Rhodes, Verlag Wiley & Sons (1998).

"Storable" in the context of this invention means a product which can be stored for up to 120 days, preferably up to 52 weeks, particularly preferably 60 months, without a substantial loss (< 5%) of methionine occurring.

Alternatively, however, the product can be absorbed on to an organic or inorganic carrier substance which is known and conventional in feedstuffs processing, for example, silicas, silicates, grits, brans, meals, starches, sugars or others, and/or mixed and stabilized with conventional thickeners or binders. Use examples and processes in this context are described in the literature (Die Mühle + Mischfüttertechnik 132 (1995) 49, page 817).

Finally, the product can be brought into a state in which it is stable to digestion by animal stomachs, in particular the stomach of ruminants, by coating processes ("coating") using film-forming agents, such as, for example, metal carbonates, silicas, silicates, alginates, stearates, starches, gums and cellulose ethers, as described in DE-C-4100920.

If the biomass is separated off during the process, further inorganic solids, for example added during the fermentation, are in general removed. In addition, the animal feedstuffs additive according to the invention comprises at least the predominant proportion of the further substances, in particular organic substances, which are formed or added and are present in solution in the fermentation broth, where these have not been separated off by suitable processes.

In one aspect of the invention, the biomass can be separated off to the extent of up to 70%, preferably up to 80%, preferably up to 90%, preferably up to 95%, and particularly



preferably up to 100%. In another aspect of the invention, up to 20% of the biomass, preferably up to 15%, preferably up to 10%, preferably up to 5%, particularly preferably no biomass is separated off.

5 These organic substances include organic by-products which are optionally produced, in addition to the L-methionine, and optionally discharged by the microorganisms employed in the fermentation. These include L-amino acids chosen from the group consisting of L-lysine, L-valine, L-threonine, L-alanine  
10 or L-tryptophan. They include vitamins chosen from the group consisting of vitamin B1 (thiamine), vitamin B2 (riboflavin), vitamin B5 (pantothenic acid), vitamin B6 (pyridoxine), vitamin B12 (cyanocobalamin), nicotinic acid/nicotinamide and vitamin E (tocopherol). They also  
15 include organic acids which carry one to three carboxyl groups, such as, acetic acid, lactic acid, citric acid, malic acid or fumaric acid. Finally, they also include sugars, such as, for example, trehalose. These compounds are optionally desired if they improve the nutritional value of the product.

20 These organic substances, including L-methionine and/or D-methionine and/or the racemic mixture D,L-methionine, can also be added, depending on requirements, as a concentrate or pure substance in solid or liquid form during a suitable process step. These organic substances mentioned can be added  
25 individually or as mixtures to the resulting or concentrated fermentation broth, or also during the drying or granulation process. It is likewise possible to add an organic substance or a mixture of several organic substances to the fermentation broth and a further organic substance or a further mixture of  
30 several organic substances during a later process step, for example granulation.

The product described above is suitable as a feedstuffs additive, i.e. feed additive, for animal nutrition.

35 The L-methionine content of the animal feedstuffs additive is conventionally 1 wt.% to 80 wt.%, preferably 2 wt.% to 80 wt.%, particularly preferably 4 wt.% to 80 wt.%, and very

particularly preferably 8 wt.% to 80 wt.%, based on the dry weight of the animal feedstuffs additive. Contents of 1 wt.% to 60 wt.%, 2 wt.% to 60 wt.%, 4 wt.% to 60 wt.%, 6 wt.% to 60 wt.%, 1 wt.% to 40 wt.%, 2 wt.% to 40 wt.% or 4 wt.% to 40 wt.% are likewise possible. The water content of the feedstuffs additive is conventionally up to 5 wt.%, preferably up to 4 wt.%, and particularly preferably less than 2 wt.%.

The invention also provides a process for the preparation of an L-methionine-containing animal feedstuffs additive from fermentation broths, which comprises the steps

- a) culture and fermentation of an L-methionine-producing microorganism in a fermentation medium;
- b) removal of water from the L-methionine-containing fermentation broth (concentration);
- c) removal of an amount of 0 to 100 wt.% of the biomass formed during the fermentation; and
- d) drying of the fermentation broth obtained according to a) and/or b) to obtain the animal feedstuffs additive in the desired powder or granule form.

If desired, one or more of the following steps can furthermore be carried out in the process according to the invention:

- e) addition of one or more organic substances, including L-methionine and/or D-methionine and/or the racemic mixture D,L-methionine, to the products obtained according to a), b) and/or c);
- f) addition of auxiliary substances chosen from the group consisting of silicas, silicates, stearates, grits and bran to the substances obtained according to a) to d) for stabilization and to increase the storability; or
- g) conversion of the substances obtained according to a) to e) into a form stable to the animal stomach, in particular rumen, by coating with film-forming agents.

The analysis of L-methionine can be carried out by ion exchange chromatography with subsequent ninhydrin derivation, as described by Spackman et al. (Analytical Chemistry, 30, (1958), 1190).

- 5 The process according to the invention is used for the fermentative preparation of amino acids, in particular L-methionine.

The following microorganisms were deposited as a pure culture on 14th June 2001 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty:

- Escherichia coli DH5 $\alpha$ mcrr/pCREmetAE as DSM 14352,
- Escherichia coli DH5 $\alpha$ mcrr/pCREmetAEY as DSM 14353.

- 15 The present invention is explained in more detail in the following with the aid of embodiment examples.

#### Example 1

Preparation of a genomic cosmid gene library from Corynebacterium glutamicum ATCC 13032

- 20 Chromosomal DNA from Corynebacterium glutamicum ATCC-13032 was isolated as described by Tauch et al. (1995, Plasmid 33:168-179) and partly cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product Description Sau3AI, Code no. 27-0913-02). The DNA fragments were
- 25 dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, Product Description SAP, Code no. 1758250). The DNA of the cosmid vector SuperCos1 (Wahl et al. (1987) Proceedings of the National Academy of Sciences USA 84:2160-2164), obtained from Stratagene (La
- 30 Jolla, USA, Product Description SuperCos1 Cosmid Vector Kit, Code no. 251301) was cleaved with the restriction enzyme XbaI (Amersham Pharmacia, Freiburg, Germany, Product Description

XbaI, Code no. 27-0948-02) and likewise dephosphorylated with shrimp alkaline phosphatase.

The cosmid DNA was then cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Code no. 27-0868-04). The cosmid DNA treated in this manner was mixed with the treated ATCC13032 DNA and the batch was treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no.27-0870-04). The ligation mixture was then packed in phages with the aid of Gigapack II XL Packing Extract (Stratagene, La Jolla, USA, Product Description Gigapack II XL Packing Extract, Code no. 200217).

For infection of the E. coli strain NM554 (Raleigh et al. 1988, Nucleic Acid Research 16:1563-1575) the cells were taken up in 10 mM MgSO<sub>4</sub> and mixed with an aliquot of the phage suspension. The infection and titering of the cosmid library were carried out as described by Sambrook et al. (1989, Molecular Cloning: A laboratory Manual, Cold Spring Harbor), the cells being plated out on LB agar (Lennox, 1955, Virology, 1:190) with 100 mg/l ampicillin. After incubation overnight at 37°C, recombinant individual clones were selected.

## Example 2

### Isolation and sequencing of the metE gene

The cosmid DNA of an individual colony was isolated with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and partly cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product Description Sau3AI, Product No. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, Product Description SAP, Product No. 1758250). After separation by gel electrophoresis, the cosmid fragments in the size range of 1500 to 2000 bp were isolated with the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany).

The DNA of the sequencing vector pZero-1, obtained from Invitrogen (Groningen, The Netherlands, Product Description Zero Background Cloning Kit, Product No. K2500-01) was cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Product No. 27-0868-04). The ligation of the cosmid fragments in the sequencing vector pZero-1 was carried out as described by Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor), the DNA mixture being incubated overnight with T4 ligase (Pharmacia Biotech, Freiburg, Germany). This ligation mixture was then electroporated (Tauch et al. 1994, FEMS Microbiol Letters, 123:343-7) into the E. coli strain DH5 $\alpha$ mcr (Grant, 1990, Proceedings of the National Academy of Sciences U.S.A., 87:4645-4649) and plated out on LB agar (Lennox, 1955, Virology, 1:190) with 50 mg/l zeocin.

The plasmid preparation of the recombinant clones was carried out with Biorobot 9600 (Product No. 900200, Qiagen, Hilden, Germany). The sequencing was carried out by the dideoxy chain termination method of Sanger et al. (1977, Proceedings of the National Academy of Sciences U.S.A., 74:5463-5467) with modifications according to Zimmermann et al. (1990, Nucleic Acids Research, 18:1067). The "RR dRhodamin Terminator Cycle Sequencing Kit" from PE Applied Biosystems (Product No. 403044, Weiterstadt, Germany) was used. The separation by gel electrophoresis and analysis of the sequencing reaction were carried out in a "Rotiphoresis NF Acrylamide/Bisacrylamide" Gel (29:1) (Product No. A124.1, Roth, Karlsruhe, Germany) with the "ABI Prism 377" sequencer from PE Applied Biosystems (Weiterstadt, Germany).

The raw sequence data obtained were then processed using the Staden program package (1986, Nucleic Acids Research, 14:217-231) version 97-0. The individual sequences of the pZero1 derivatives were assembled to a continuous contig. The computer-assisted coding region analysis was prepared with the XNIP program (Staden, 1986, Nucleic Acids Research, 14:217-231).

The resulting nucleotide sequence is shown in SEQ ID No. 1. Analysis of the nucleotide sequence showed an open reading frame of 2235 base pairs, which was called the metE gene. The metE gene codes for a protein of 745 amino acids.

### 5 Example 3

Preparation of the strains *C. glutamicum* ATCC13032/pCREmetA and ATCC13032/pCREmetAE

#### 3.1 Amplification of the genes metA and metE

10 From the strain ATCC13032, chromosomal DNA was isolated by the method of Eikmanns et al. (Microbiology 140: 1817 -1828 (1994)). Starting from the nucleotide sequences of the methionine biosynthesis genes metA (gene library Accession Number AF052652) and metE (SEQ ID No. 1) of *C. glutamicum* ATCC13032, the following oligonucleotides were chosen for the polymerase chain reaction (PCR) (see SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5 and SEQ ID No. 6):

metA-EVP5:

5'- AGAACGAATTCAAAGGAGGACAACCATGCCCACCCTCGCGC -3'

metA-EVP3:

20 5'- GTCGTGGATCCCCTATTAGATGTAGAACTCG -3'

metE-EVP5:

5'-GGCTCAAAGATCTAAAGGAGGACAACCATGACTTCCAACCTTTTCTTC -3'

metE-EVP3:

5'- GGTCCTGTCGACGGTACCATTTAGATAGTTGCTCCGATT -3'

25 The primers shown were synthesized by MWG-Biotech AG (Ebersberg, Germany) and the PCR reaction was carried out by the standard PCR method of Innis et al. (PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press) with Pwo-Polymerase from Roche Diagnostics GmbH (Mannheim, Germany). With the aid of the polymerase chain reaction, the  
30 primers allow amplification of a DNA fragment 1161 bp in size,

which carries the metA gene, and a DNA fragment 2286 bp in size, which carries the metE gene.

Furthermore, the primer metA-EVP5 contains the sequence for the cleavage site of the restriction endonuclease EcoRI, the primer metA-EVP3 the cleavage site of the restriction endonuclease BamHI, the primer metE-EVP5 the cleavage site of the restriction endonuclease BglII and the primer metE-EVP3 the cleavage site of the restriction endonuclease SalI, which are marked by underlining in the nucleotide sequence shown above.

The metA fragment 1161 bp in size was cleaved with the restriction endonucleases EcoRI and BamHI, and the metE fragment 2286 bp in size was cleaved with the restriction endonucleases BglII and SalI. The two batches were separated by gel electrophoresis and the fragments metA (approx. 1150 bp) and metE (approx. 2270 bp) were then isolated from the agarose gel with the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany).

### 3.2 Cloning of metA in the vector pZ8-1

The E. coli - C. glutamicum shuttle expression vector pZ8-1 (EP 0 375 889) was used as the base vector for the expression.

DNA of the plasmid pZ8-1 was cleaved completely with the restriction enzymes EcoRI and BamHI and then dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, Product Description SAP, Product No. 1758250).

The metA fragment approx. 1150 bp in size isolated from the agarose gel in example 3.1 and cleaved with the restriction endonucleases BamHI and EcoRI was mixed with the vector pZ8-1 prepared in this way and the batch was treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no.27-0870-04).

The ligation batch was transformed in the E. coli strain DH5αmcr (Hanahan, In: DNA cloning. A Practical Approach. Vol.

I. IRL-Press, Oxford, Washington DC, USA). Selection of plasmid-carrying cells was made by plating out the transformation batch on LB agar (Lennox, 1955, Virology, 1:190) with 50 mg/l kanamycin. After incubation overnight at 37°C, recombinant individual clones were selected. Plasmid DNA was isolated from a transformant with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and checked by restriction cleavage. The resulting plasmid was called pCREmetaA.

### 3.3 Cloning of metE in the vector pCREmetaA

The plasmid pCREmetaA described in example 3.2 was cleaved completely with the restriction enzymes BamHI and SalI and then dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, Product Description SAP, Product No. 1758250).

The metE fragment approx. 2270 bp in size obtained in example 3.1 by means of the polymerase chain reaction and cleaved with the restriction endonucleases BglII and SalI was mixed with the vector pCREmetaA prepared in this way. The batch was treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no.27-0870-04).

The ligation batch was transformed in the E. coli strain DH5 $\alpha$ mcr (Hanahan, In: DNA cloning. A Practical Approach. Vol. I. IRL-Press, Oxford, Washington DC, USA). Selection of plasmid-carrying cells was made by plating out the transformation batch on LB agar (Lennox, 1955, Virology, 1:190) with 50 mg/l kanamycin. After incubation overnight at 37°C, recombinant individual clones were selected. Plasmid DNA was isolated from a transformant with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and checked by restriction cleavage. The resulting plasmid was called pCREmetaAE. It is shown in figure 1. The strain E. coli DH5 $\alpha$ mcr/pCREmetaAE was deposited as a pure culture on 14th June



2001 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty as DSM 14352.

5 3.4 Preparation of the strains *C. glutamicum*  
ATCC23032/pCREmetA and ATCC13032/pCREmetAE

The vectors pCREmetA and pCREmetAE obtained in example 3.2 and 3.3 were electroporated in the strain *C. glutamicum* ATCC13032 using the electroporation method described by Liebl et al.  
10 (FEMS Microbiology Letters, 53:299-303 (1989)). Selection of the plasmid-carrying cells took place on LBHIS agar comprising 18.5 g/l brain-heart infusion broth, 0.5 M sorbitol, 5 g/l Bacto-tryptone, 2.5 g/l Bacto-yeast extract, 5 g/l NaCl and 18 g/l Bacto-agar, which had been supplemented with 25 mg/l kanamycin. Incubation was carried out for 2 days at 33°C.  
15

Plasmid DNA was isolated from in each case one transformant by conventional methods (Peters-Wendisch et al., 1998, Microbiology 144, 915-927) and checked by restriction cleavage. The resulting strains were called ATCC13032/pCREmetA and ATCC13032/pCREmetAE.  
20

Example 4

Preparation of L-methionine with the strain *C. glutamicum* ATCC13032/pCREmetAE

The *C. glutamicum* strains ATCC13032/pCREmetA and  
25 ATCC13032/pCREmetAE obtained in example 3 were cultured in a nutrient medium suitable for the production of methionine and the methionine content in the culture supernatant was determined.

For this, the strains were first incubated on an agar plate  
30 with the corresponding antibiotic (brain-heart agar with kanamycin (50 mg/l)) for 24 hours at 33°C. Starting from this agar plate culture, in each case a preculture was seeded (10 ml medium in a 100 ml conical flask). The complete medium CgIII was used as the medium for the precultures.

### Medium Cg III

NaCl 2.5 g/l

Bacto-Peptide 10 g/l

Bacto-Yeast extract 10 g/l

Glucose (autoclaved separately) 2% (w/v)

The pH was brought to pH 7.4

Kanamycin (25 mg/l) was added to this. The preculture was incubated for 16 hours at 33°C at 240 rpm on a shaking machine. In each case a main culture was seeded from these precultures such that the initial OD (660 nm) of the main cultures was 0.1. Medium MM was used for the main cultures.

# Medium MM

CSL (corn steep liquor)	5 g/l
MOPS (morpholinopropanesulfonic acid)	20 g/l
Glucose (autoclaved separately)	50 g/l
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	25 g/l
KH <sub>2</sub> PO <sub>4</sub>	0.1 g/l
MgSO <sub>4</sub> * 7 H <sub>2</sub> O	1.0 g/l
CaCl <sub>2</sub> * 2 H <sub>2</sub> O	10 mg/l
FeSO <sub>4</sub> * 7 H <sub>2</sub> O	10 mg/l
MnSO <sub>4</sub> * H <sub>2</sub> O	5.0mg/l
Biotin (sterile-filtered)	0.3 mg/l
Thiamine * HCl (sterile-filtered)	0.2 mg/l
CaCO <sub>3</sub>	25 g/l

The CSL, MOPS and the salt solution were brought to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions were then added, as well as the CaCO<sub>3</sub> autoclaved in the dry state.

Culturing is carried out in a 10 ml volume in 100 ml conical flasks with baffles. Kanamycin (25 mg/l) was added. Culturing was carried out at 33°C and 80% atmospheric humidity.

After 72 hours, the OD was determined at a measurement wavelength of 660 nm with a Biomek 1000 (Beckmann Instruments GmbH, Munich). The amount of methionine formed was determined with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivation with ninhydrin detection.

The result of the experiment is shown in Table 1.

Table 1

Strain	OD (660 nm)	Methionine mg/l
ATCC13032/pCREmetA	12.3	6.6
ATCC13032/pCREmetAE	14.3	15.3

### Example 5

Preparation of the strain *C. glutamicum* ATCC13032/pCREmetAEY

#### 5 5.1 Amplification of the metY gene

From the strain ATCC13032, chromosomal DNA was isolated by the method of Eikmanns et al. (Microbiology 140: 1817 -1828 (1994)). Starting from the nucleotide sequence of the methionine biosynthesis gene metY (DE: 10043334.0) of *C. glutamicum* ATCC13032, the following oligonucleotides were chosen for the polymerase chain reaction (PCR) (see SEQ ID No. 7 and SEQ ID No. 8):

metY-EVP5:

5'- CTAATAAGTCGACAAAGGAGGACAACCATGCCAAAGTACGAC -3'

metY-EVP3:

5'- GAGTCTAATGCATGCTAGATTGCAGCAAAGCCG -3'

The primers shown were synthesized by MWG-Biotech AG (Ebersberg, Germany) and the PCR reaction was carried out by the standard PCR method of Innis et al. (PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press) with Pwo-Polymerase from Roche Diagnostics GmbH (Mannheim, Germany). With the aid of the polymerase chain reaction, the primers allow amplification of a DNA fragment 1341 bp in size, which carries the metY gene.

Furthermore, the primer metY-EVP5 contains the sequence for the cleavage site of the restriction endonuclease SalI and the

primer metY-EVP3 the cleavage site of the restriction endonuclease NsiI, which are marked by underlining in the nucleotide sequence shown above.

The metY fragment 1341 bp in size was cleaved with the restriction endonucleases SalI and NsiI. The batch was separated by gel electrophoresis and the fragment metY (approx. 1330 bp) was then isolated from the agarose gel with the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany).

## 5.2 Cloning of metA and metY in the vector pZ8-1

The plasmid pCREmetA described in example 3.2 was cleaved completely with the restriction enzymes SalI and PstI and then dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, Product Description SAP, Product No. 1758250).

The metY fragment approx. 1330 bp in size isolated from the agarose gel in example 5.1 and cleaved with the restriction endonucleases SalI and NsiI was mixed with the vector pCREmetA prepared in this way and the batch was treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no.27-0870-04).

The ligation batch was transformed in the E. coli strain DH5 $\alpha$ mcr (Hanahan, In: DNA cloning. A Practical Approach. Vol. I. IRL-Press, Oxford, Washington DC, USA). Selection of plasmid-carrying cells was made by plating out the transformation batch on LB agar (Lennox, 1955, Virology, 1:190) with 50 mg/l kanamycin. After incubation overnight at 37°C, recombinant individual clones were selected. Plasmid DNA was isolated from a transformant with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and checked by restriction cleavage. The resulting plasmid was called pCREmetAY.

### 5.3 Cloning of metE in the vector pCREmetAY

The plasmid pCREmetAY described in example 5.2 was cleaved completely with the restriction enzymes BamHI and SalI and then dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, Product Description SAP, Product No. 1758250).

The metE fragment approx. 2270 bp in size obtained in example 3.1 by means of the polymerase chain reaction and cleaved with the restriction endonucleases BglII and SalI was mixed with the vector pCREmetAY prepared in this way. The batch was treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no.27-0870-04).

The ligation batch was transformed in the E. coli strain DH5 $\alpha$ mc<sup>r</sup> (Hanahan, In: DNA cloning. A Practical Approach. Vol. I. IRL-Press, Oxford, Washington DC, USA). Selection of plasmid-carrying cells was made by plating out the transformation batch on LB agar (Lennox, 1955, Virology, 1:190) with 50 mg/l kanamycin. After incubation overnight at 37°C, recombinant individual clones were selected. Plasmid DNA was isolated from a transformant with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and checked by restriction cleavage. The resulting plasmid was called pCREmetAEY. It is shown in figure 2. The strain E. coli DH5 $\alpha$ mc<sup>r</sup>/pCREmetAEY was deposited as a pure culture on 14th June 2001 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty as DSM 14353.

### 5.4 Preparation of the strain C. glutamicum ATCC13032/pCREmetAEY

The vector pCREmetAY obtained in example 5.3 was electroporated in the strain C. glutamicum ATCC13032 using the electroporation method described by Liebl et al. (FEMS Microbiology Letters, 53:299-303 (1989)). Selection of

plasmid-carrying cells took place on LBHIS agar comprising 18.5 g/l brain-heart infusion broth, 0.5 M sorbitol, 5 g/l Bacto-tryptone, 2.5 g/l Bacto-yeast extract, 5 g/l NaCl and 18 g/l Bacto-agar, which had been supplemented with 25 mg/l kanamycin. Incubation was carried out for 2 days at 33°C.

Plasmid DNA was isolated from a transformant by conventional methods (Peters-Wendisch et al., 1998, Microbiology 144, 915-927) and checked by restriction cleavage. The resulting strain was called ATCC13032pCREmetAEY.

## 10 Example 6

Fermentative preparation of L-methionine with the strain ATCC13032/pCREmetAEY

15 The strain *C. glutamicum* ATCC13032/pCREmetAEY constructed by the process described in example 4 was cultured in a nutrient medium suitable for the production of methionine and the methionine content in the culture supernatant was determined.

20 For this, the strain was first incubated on an agar plate with the corresponding antibiotic (brain-heart agar with kanamycin (25 mg/l)) for 24 hours at 33°C. Starting from this agar plate culture, a preculture was seeded (10 ml medium in a 100 ml conical flask). The medium MM-1 was used as the medium for the preculture.

### Medium MM-1

CSL (corn steep liquor)	5 g/l
MOPS (morpholinopropanesulfonic acid)	20 g/l
Glucose (autoclaved separately)	50g/l
Salts:	
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	25 g/l
KH <sub>2</sub> PO <sub>4</sub>	0.1 g/l

MgSO <sub>4</sub> * 7 H <sub>2</sub> O	1.0 g/l
CaCl <sub>2</sub> * 2 H <sub>2</sub> O	10 mg/l
FeSO <sub>4</sub> * 7 H <sub>2</sub> O	10 mg/l
MnSO <sub>4</sub> * H <sub>2</sub> O	5.0mg/l
Biotin (sterile-filtered)	0.01 mg/l
Vitamin B12 (sterile-filtered)	0.02 mg/l
Thiamine * HCl (sterile-filtered)	0.2 mg/l
CaCO <sub>3</sub>	25 g/l

The CSL, MOPS and the salt solution were brought to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions were then added, as well as the CaCO<sub>3</sub> autoclaved in the dry state. Kanamycin (25 mg/l) was added to this.

The preculture was incubated for 16 hours at 33°C at 240 rpm on a shaking machine and then used as the inoculum for the main culture in the fermenter. To establish an optical density(at 660 nm) of 1.0 as the starting value for the main culture in the fermenter, the corresponding amount of culture broth was transferred from the preculture.

The medium MM-2, which has the following composition, was used for the main culture:

#### Medium MM-2

CSL (corn steep liquor)	5 g/l
Glucose (autoclaved separately)	50g/l
Salts:	
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	25 g/l
KH <sub>2</sub> PO <sub>4</sub>	0.1 g/l



MgSO <sub>4</sub> * 7 H <sub>2</sub> O	1.0 g/l
CaCl <sub>2</sub> * 2 H <sub>2</sub> O	10 mg/l
FeSO <sub>4</sub> * 7 H <sub>2</sub> O	10 mg/l
MnSO <sub>4</sub> * H <sub>2</sub> O	5.0mg/l
Biotin (sterile-filtered)	0.01 mg/l
Vitamin B12 (sterile-filtered)	0.02 mg/l
Thiamine * HCl (sterile-filtered)	0.2 mg/l
Antifoam (Structol)	0.5 g/l

All the components of the medium were initially introduced directly into the fermenter, dissolved in water and then sterilized by means of heat (121°C, 20 minutes). Only the glucose was prepared in a stock solution of 50 wt.% and sterilized separately (also 121°C, 20 minutes). Biotin or thiamine were sterile-filtered and added under aseptic conditions directly before the start of fermentation.

Culturing was carried out by the batch process in a bioreactor with a working volume of 0.5 L (Multifermenter SIXFORS from Infors GmbH, Bodmingen, Switzerland). After addition of the inoculum, the starting volume in the fermenter was 0.4 L in total. Further culturing was carried out under constant aeration (0.1 vvm ("volume per volume per minute") and stirring at 33°C and a pH of 7.0. Correction or adjustment of the pH was carried out with a 5% NH<sub>4</sub>OH solution. The set value for the concentration of dissolved oxygen in the fermentation medium was regulated at 40% and adjusted via the stirrer speed at a constant rate of aeration.

After 48 hours the process was ended and the optical density (OD) of the culture suspension was determined with an LP2W photometer from Dr. Lange (Berlin, Germany) at a measurement wavelength of 660 nm. The concentration of L-methionine formed was determined with an amino acid analyzer from Eppendorf-

BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivation with ninhydrin detection.

An optical density in the final sample of 31.7 and a concentration of L-methionine of 39.0 mg per liter could be determined by the methods described above as the result.

#### Example 7

Preparation of biomass-free broth containing L-methionine

The biomass was first separated off from a fermentation broth comprising L-methionine prepared by the process of example 6 and comprising about 39 mg/l L-methionine. For this, 0.5 l of the above-mentioned fermentation broth was centrifuged with a laboratory centrifuge of the Biofuge-Stratos type from Heraeus (Düsseldorf, Germany) for 20 minutes at 4,000 rpm and the supernatant from the centrifugation was then purified further by cross-flow ultrafiltration with an MRC polymer membrane of 30kD in an ultrafiltrations unit from ICT GmbH (Bad Homburg, Germany).

#### Example 8

Preparation of a biomass-free product comprising L-methionine from a fermentation broth

The biomass was first separated off from a fermentation broth comprising L-methionine prepared by the process as described under example 6 and comprising about 39.0 mg/l L-methionine. For this, the fermenter contents of the above-mentioned fermentation broth were centrifuged and subjected to ultrafiltration as described in example 7.

23.7 g pure L-methionine (>99%; MERCK, Darmstadt, Germany) were then added batchwise to 300 g of the biomass-free filtrate, while stirring, in order to establish the desired content of L-methionine in the product. The suspension comprising L-methionine treated in this way was then mixed with 150 g water, with further stirring, to improve the working-up properties.

A portion of the suspension improved in this way was then lyophilized in a freeze-dryer of the type LYOVAC GT 2 from Leybold (Cologne, Germany). The product comprising L-methionine prepared in this manner had a content of 70 wt.% L-methionine and was free-flowing.

The remaining portion of the suspension improved in this way was treated by means of spray drying in a laboratory spray dryer of the Büchi-190 type from Büchi-Labortechnik GmbH (Constance, Germany) at an intake temperature of 170°C, a starting temperature of 105°C, a pressure difference of -40 mbar and an air flow rate of 600 NL/h. The product comprising L-methionine prepared in this manner had a content of 70 wt.% L-methionine and was free-flowing.

#### Example 9

Preparation of a biomass-containing product comprising L-methionine from a fermentation broth

From a fermentation broth comprising L-methionine prepared by the process of example 6 and comprising about 39 mg/l L-methionine, 23.7 g pure L-methionine (>99%; MERCK, Darmstadt, Germany) was first added batchwise, while stirring, in order to establish the desired content of L-methionine in the product. The fermentation broth treated in this way was then mixed with 150 g water, with further stirring, to improve the working-up properties.

A portion of this biomass-containing broth was then lyophilized in a freeze-dryer of the type LYOVAC GT 2 from Leybold (Cologne, Germany). The product comprising L-methionine prepared in this way had a content of 65 wt.% L-methionine and was free-flowing.

The remaining portion of the biomass-containing broth was treated by means of spray drying in a laboratory spray dryer of the Büchi-190 type from Büchi-Labortechnik GmbH (Constance, Germany) at an intake temperature of 170°C, a starting temperature of 105°C, a pressure difference of -40 mbar and an

air flow rate of 600 NL/h. The product comprising L-methionine prepared in this way had a content of 65 wt.% L-methionine and was free-flowing.

Brief Description of the Figures:

- Figure 1: Plasmid pCREmetAE
- Figure 2: pCREmetAEY

The abbreviations used in the figures have the following meaning:

Km: Resistance gene for kanamycin

metE: metE gene of *C. glutamicum*

metY: metY gene of *C. glutamicum*

metA: metA gene of *C. glutamicum*

Ptac: tac promoter

rrnB-T1T2: Terminator T1T2 of the rrnB gene of *E. coli*

rep: Plasmid-coded replication origin for *C. glutamicum*  
(of pHM1519)

BamHI: Cleavage site of the restriction enzyme BamHI

EcoRI: Cleavage site of the restriction enzyme EcoRI

Sall: Cleavage site of the restriction enzyme Sall

This disclosure is based on priority documents DE 100 38 023.9, DE 101 09 689.5 and US 60/294,250, each incorporated by reference.

Obviously, numerous modifications of the invention are possible in view of the above teachings. Therefore, within the scope of the appended claims, the invention may be practiced otherwise than as specifically described herein.

# SEQUENCE PROTOCOL

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Met Thr Ser Asn Phe Ser Ser Thr Val Ala Gly Leu

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Pro Arg Ile Gly Ala Lys Arg Glu Leu Lys Phe Ala Leu Glu Gly Tyr

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tgg aat gga tca att gaa ggt cgc gaa ctt gcg cag acc gcc cgc caa 448

Trp Asn Gly Ser Ile Glu Gly Arg Glu Leu Ala Gln Thr Ala Arg Gln

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